

Bioconjugates of luminescent CdSe-ZnS quantum dots with engineered recombinant proteins: Novel self-assembled tools for biosensing

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ABSTRACT

Colloidal semiconductor quantum dots (QDs) are luminescent nanoparticles with size-dependent emission spectra spanning a wide range of wavelengths in the visible and near IR. This property, as well as their higher resistance to photo-degradation compared to organic dye labels, makes QDs potentially suitable for certain biomolecule tagging and multiplexing applications. We describe an electrostatic self-assembly approach for conjugating highly luminescent colloidal CdSe-ZnS core-shell QDs with engineered two-domain recombinant proteins to form conjugates for sensing and imaging applications. The design, preparation, and characterization of high quantum yield IgG antibody-binding protein G bioconjugates using luminescence, electrophoretic gel shift, and affinity assays is reported.

INTRODUCTION

Fluorescent labeling of biological materials using organic dyes has achieved a high level of sophistication, and is widely used in single and multiplex detection and in biological imaging [1]. Nonetheless, this technique is subject to several limitations due to the characteristics of organic dyes. Organic labels often have narrow excitation spectra and broad emission bands with red spectral tails, which makes simultaneous evaluation of different probes difficult due to spectral overlap. Many organic dyes also exhibit low resistance to photodegradation [2]. Luminescent colloidal semiconductor nanocrystals (quantum dots, QDs), which have inorganic cores and surfaces derivatizable with a variety of functional groups, including water-compatible groups, have the potential to overcome some of the problems encountered by organic dyes. These nanoparticles combine size-dependent tunable photoluminescence and high quantum yield with high resistance to photo-degradation compared to organic labels. These properties make QDs potentially useful in numerous fluorescence tagging applications.

CdSe-ZnS core-shell QDs are tunable fluorophores with narrow emission bands (Full width at half maximum, FWHM, of ~ 30-45 nm) spanning the visible spectrum, and they have broad absorption spectra, a property that allows simultaneous excitation of several particle sizes at a single wavelength [3-7]. These QDs can be detected at concentrations comparable to organic dyes by conventional fluorescence methods, and individual bioconjugated QDs are observable by confocal microscopy [8].

We have developed a novel conjugation strategy, based on electrostatic interaction between negatively charged dihydrolipoic acid (DHLA) capped CdSe-ZnS core-shell QDs and engineered bifunctional recombinant proteins consisting of positively charged leucine zipper interaction domains genetically fused with functional N-terminal domains [9]. Here we describe an example of this strategy for conjugate formation, namely the preparation and characterization of CdSe-ZnS QDs conjugated with the immunoglobulin G (IgG)-binding B2 domain of streptococcal protein G (PG) modified with an electrostatic interaction leucine zipper module.

MATERIALS PREPARATION AND EXPERIMENTAL DETAILS

The present nanocrystalline CdSe cores were prepared using high temperature solution synthesis followed by a ZnS-overcoating layer and trioctyl phosphine/trioctyl phosphine oxide mixture (TOP/TOPO) capping as described in previous works [3,6,7]. To make the core-shell QDs water-compatible, their TOP/TOPO-caps were exchanged with dihydrolipoic acid (DHLA) groups by suspending purified and dried nanocrystals in DHLA [10] and heating to ~ 60-70°C. Subsequent deprotonation using potassium-tert-butoxide (K-t-butoxide) permitted dispersion of the new materials in water. The QD material was separated from excess hydrolyzed K-t-butoxide and residual TOPO/TOPO by centrifugation and repetitive concentration/dilution using centrifugal ultrafiltration, and then were redispersed in deionized water. This procedure allowed preparation of stable aqueous CdSe-ZnS/DHLA nanocrystal solutions that are stable and have photoluminescence quantum yields (PL QY) of ~ 10-20%.

The two-domain protein G-basic zipper (PG-zb) fusion protein (shown schematically in Fig. 1) was constructed using gene assembly and cloning. Polymerase chain reaction (PCR) was employed to amplify the B2 IgG binding domain of streptococcal protein G (Protein G) and to introduce cloning sites to be used for producing plasmid pBadG [11]. The initial purification

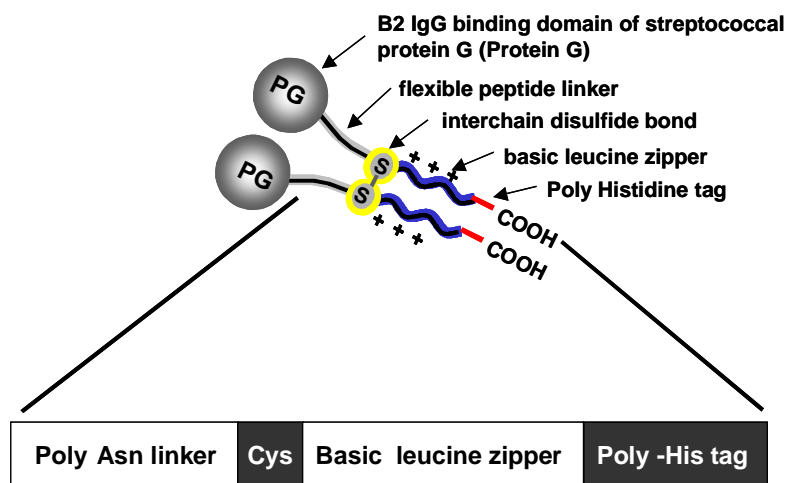


Fig. 1. Cartoon of a PG-zb fusion protein showing the sequences of the C-terminal basic leucine interaction domain.

step of the PG-zb protein from *E. coli* Top 10 (Invitrogen) cells was carried out under denaturing conditions using Ni-NTA agarose resin as described by the manufacturer (Qiagen) [12]. After the denatured protein was loaded onto the column and washed with denaturing buffer, protein re-folding was carried out on the column during extensive washing with PBS, followed by elution with 50mM NaH₂PO₄, 300 mM NaCl and 250 mM imidazole at pH 6.3. A second purification step through SP Sepharose ion exchange resin was added to separate monomer from the majority dimer product. The sample was loaded onto the column in PBS buffer, and eluted with an increasing NaCl gradient up to 1M NaCl; dimer product eluted at about 80% of the 1M NaCl.

Conjugation of the two-domain PG-zb fusion proteins and DHLA-capped CdSe-ZnS QDs was carried out in 10-mM sodium borate pH 9. Absorption and photoluminescence spectra were measured using 10-mm optical path length cells (Spectrocell) using a diode array UV-VIS spectrometer (Hewlett-Packard) and a SPEX Fluorolog-3 (ISA), respectively.

A direct binding assay was employed to examine binding of PG-zb conjugated QDs to IgG. Goat IgG (10 µg/ml) was incubated overnight in wells of black microtiter plates (FluoroNunc module Maxisorp surface, Nunc). After the IgG coated wells were washed and blocked with 4% powdered non-fat milk, PG-zb conjugated QDs were incubated for 1-2 hr in the IgG adsorbed wells or control wells to which no IgG had been adsorbed. The wells were then washed with borate buffer and the fluorescence was measured utilizing a Spectra Fluor Plus microtiter plate reader (Tecan). A 25-nm band pass filter was used for excitation at 360 nm and a long pass filter with a cut-off at 530 nm was used for monitoring PL intensity.

For QD/PG-zb gel-shift electrophoresis experiments, 30 µL samples containing ~ 11 picomoles of nanoparticles and variable protein:QD molar ratios were prepared. A 6x loading buffer containing 0.2% each of bromophenol blue (Sigma) and xylene cyanole FF (Kodak) tracking dyes and 15% Ficoll (Sigma) was added to the 30 µL samples before they were loaded into the wells of a 1.2% agarose gel in 1xTBE buffer (tris/borate EDTA) [13], and run at 82 volts for 1 hour at ambient temperature. The fluorescent QD bands were visualized using UV epi-illumination excitation at 365 nm, and a long pass cut-off filter at 523 nm using a Kodak Image Station 440.

RESULTS AND DISCUSSION

Mixing the DHLA-capped QDs and PG-zb in borate buffer pH 9, yielded QD/protein bioconjugates free of any obvious macroscopic aggregates. The conjugates retain the absorption and photoluminescence properties of the starting CdSe-ZnS nanocrystals. Moreover, conjugating DHLA-capped CdSe-ZnS QDs to PG-zb enhanced the PL of the conjugates compared to unconjugated nanocrystals. Fig. 2 shows the relative change in the PL integrated intensity with an increasing molar ratio of PG-zb to QDs (at a fixed nanocrystal molar concentration). A net enhancement in the PL intensity is measured for QD-conjugates compared with unconjugated nanocrystals when the ratio PG-zb:QD increases up to ~ 20, before saturation at higher ratios. The PL quantum efficiency increases from ~ 12% for unconjugated CdSe-ZnS QDs to ~ 15-25% for conjugates in basic solutions. This PL enhancement effect was also observed when the *E. coli* maltose-binding protein (MBP) appended with an identical leucine zipper electrostatic interaction domain was used to form QD/MBP-zb bioconjugates [8].

Electrophoretic mobility shift of QDs upon bioconjugation confirms stable electrostatic interaction between the bifunctional PG-zb and the DHLA-capped nanocrystals, as shown in

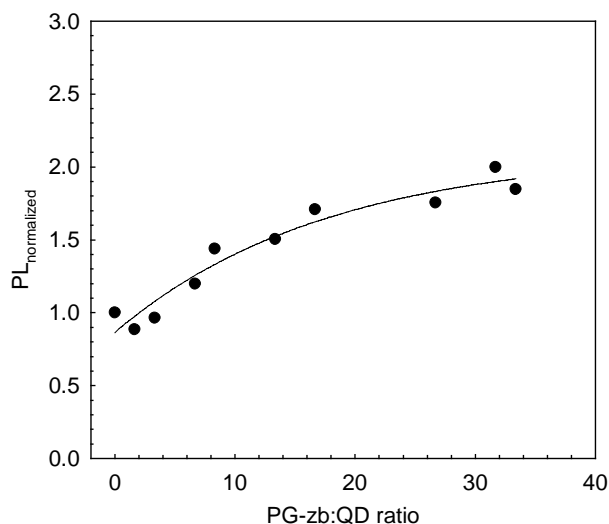


Fig. 2. PL enhancement upon bioconjugate formation at increasing PG-zb:QD ratios (data normalized against unconjugated QDs). Experiments were carried out in 10-mM sodium borate, 10-mM EDTA at pH 9.

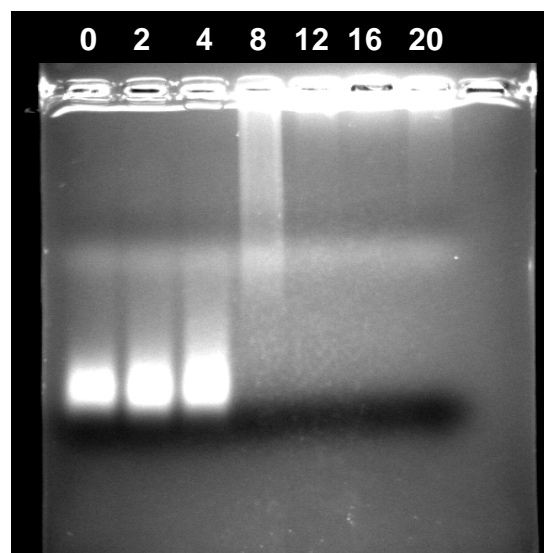


Fig. 3. Gel shift as a function of PG-zb:QD molar ratio for a fixed nanocrystal concentration. The ratio of PG-zb:QD used for each solution is shown on top of the picture. The conjugates were prepared in 10-mM sodium borate, 10-mM EDTA at pH9.

Fig. 3. Due to a net negative charge from the DHLA cap on their surfaces, unconjugated QDs migrate towards the positive electrode in conventional agarose gel electrophoresis, manifesting in luminescent bands in the middle of the gel slab, observable upon UV excitation (lane 1). The dark stripe below the fluorescent bands in lanes 1-3 and observed in every sample lane is due to the bromophenol blue dye in the 6x loading buffer used. Incubating QDs in an excess of PG-zb (PG-zb:QD molar ratio of 12 or above) resulted in nearly complete retardation of their movement, where the florescent bands were observed in or very near the loading wells of the gel (lanes 4-7). This effect is likely due to association of PG-zb molecules with QDs, eclipsing their negative surface charge and rendering the conjugated QDs incapable of migrating into the gel. Furthermore, the additional mass from several molecules of protein G, upon their association with each nanocrystal, would likewise reduce QD eletrophoretic mobility, keeping the bands near the loading wells. Although low concentrations of PG-zb (at protein-to-QD molar ratio of 2 or 4) failed to induce a substantial shift in the QD band, an increase in bandwidth and its intensity relative to unconjugated QDs (compare lanes 2 and 3 to lane 1), accompanied by an apparent smearing above the bands, suggests that some level of protein-QD interaction is present. The smearing in this case may be attributed to heterogeneous and partial masking of the QD surface charges due to conjugation with protein G, so that the small fraction of larger size conjugates migrate more slowly than the majority of QDs in the reaction. At the intermediate PG-zb:QD molar ratio of 8 (lane 4), a more widely distributed low intensity smear close to the loading well was observed, suggesting that only a small number of conjugates has migrated into the gel. This may represent the reverse scenario of the case discussed above (PG-zb:QD molar ratio of 4), where only a small fraction of heterogeneous and partially unmasked QD/protein surfaces migrated slowly into the gel. The data shown in Fig. 3

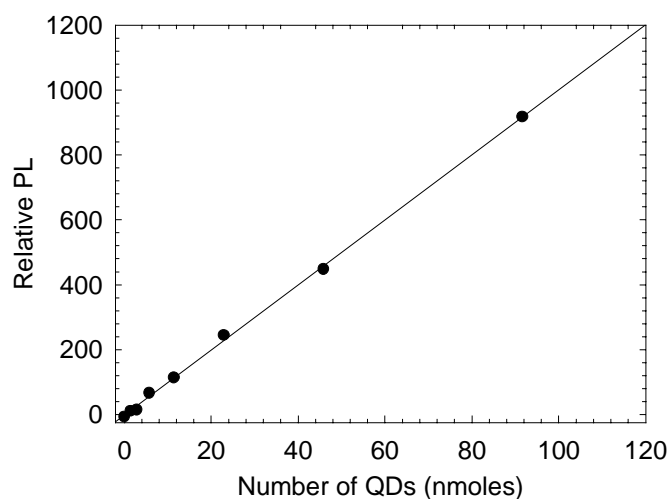


Fig. 4. Direct binding of QD/PG-zb to immobilized IgG. Relative PL intensity measured on the plate reader (after rinsing) versus number of QDs added to wells. Experiments were carried out in buffered solutions at pH 9.

indicate that this reaction resulted in a heterogeneous population of bioconjugates, where the products consisted of a mixture of individual QDs, each conjugated with a variable number of PG-zb molecules. The data shown in Figures 2 and 3 also provide evidence that each particle binds to several PG-zb molecules, similar to what was observed using MBP-zb to coat the QDs [8].

The QD/PG-zb bioinorganic conjugates retain their ability to bind IgG antibodies as demonstrated by a direct binding assay. QD/PG-zb conjugates at increasing concentrations were incubated in the wells of a microtiter plate on which IgG had been adsorbed. Figure 4 shows the relative PL intensity after washing the wells as a function of the QD/PG-zb input concentration. The measured signal increased linearly through the concentration range, indicating the QD/PG-zb conjugates effectively bind to the adsorbed IgG molecules. Saturation was not reached under these experimental conditions; however when all Fc binding sites are occupied with QD/PG-zb, the signal should stay constant regardless of the addition of higher concentrations of QD-conjugates. This experiment demonstrates that the Fc region of much of the adsorbed IgG remains accessible to the QD/PG-zb conjugate, and that conjugate formation has no serious effects on the IgG binding ability of the functional protein G domain of the fusion protein.

The above results demonstrate effective non-covalent conjugation, driven by electrostatic self-assembly, between DHLA-capped CdSe-ZnS QDs and PG-zb. The new bioinorganic conjugates conserve both the properties of the QDs (absorption and PL) and PG (binding to IgG).

The present technique for conjugating QDs with biological molecules complements previously reported methods that employed either avidin-biotin technology or covalent cross-linking using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) [14] condensation to conjugate the nanoparticles to either Actin fibers or IgG, respectively [15,16]. Nevertheless, the electrostatic self-assembly approach is fundamentally different from those using covalent conjugation techniques, as binding is driven by electrostatic attractions between interaction

modules, added to the protein by molecular design, and QDs. Electrostatically driven conjugate formation using this approach should be directly applicable to additional charged systems, such as metal nanoparticles, polymeric colloids, and other natural bioassemblies.

CONCLUSION

We have described a conjugation approach based on electrostatic self-assembly to form stable QD-bioconjugates, and have demonstrated that chimeric PG-zb can be used to surface-modify QDs. The resulting QD/PG-zb conjugates retain the PL properties of the CdSe-ZnS core-shell quantum dots as well as the IgG binding activity of protein G. Developing functional QD/PG-zb conjugates represents an important step in using QDs as fluorescent tags in immunoassays.

Use of electrostatic self-assembly to bring together charged nanoparticles and recombinant fusion proteins is a general approach. It should be applicable to labeling of other size-tunable semiconductor and inorganic colloidal particles, to create an array of bioconjugates for use in biosensing and diagnostics applications.

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REFERENCES

1. E. Schröck, S. du Manoir, T. Veldman, B. Schoell, J. Wienberg, M. A. Ferguson-Smith, Y. Ning, D. H. Ledbetter, I. Bar-Am, D. Soenksen, Y. Garini and T. Ried, *Science* **273**, 494 (1996).
2. G. T. Hermanson, *Bioconjugate Techniques*, Academic Press, London (UK), 1996 (chapter 8).
3. C. B. Murray, D. J. Norris and M. G. Bawendi, *J. Am. Chem. Soc.* **115**, 8706 (1993).
4. H. Mattoussi, L. H. Radzilowski, B. O. Dabbousi, E. L. Thomas, M. G. Bawendi and M. F. Rubner, *J. Appl. Phys.* **83**, 7965 (1998).
5. J. Rodriguez-Viejo, H. Mattoussi, J. R. Heine, M. K. Kuno, J. Michel, M. G. Bawendi and K. F. Jensen, *J. Appl. Phys.* **87**, 8526 (2000).
6. M. A. Hines and P. Guyot-Sionnest, *P. J. Phys. Chem.* **100**, 468 (1996).
7. B. O. Dabbousi, J. Rodriguez-Viejo, F. V. Mikulec, J. R. Heine, H. Mattoussi, R. Ober, K. F. Jensen and M. G. Bawendi, *J. Phys. Chem.* **101**, 9463 (1997).
8. H. Mattoussi, J. M. Mauro, E. R. Goldman, G. P. Anderson, V. C. Sundar, F. V. Mikulec and M. G. Bawendi, *J. Am. Chem. Soc.* **122**, 12142 (2000).
9. E. K. O'Shea, K. J. Lumb and P. S. Kim, *Curr. Biol.* **3**, 658 (1993).
10. L. C. Gunzalus, L. S. Barton and W. Gruber, *J. Am. Chem. Soc.* **78**, 1763 (1956).
11. J. M. Mauro, L. K. Cao, L. M. Kondracki, S. E. Walz, J. R. Campbell Jr., *Analytical Biochemistry* **235**, 61-72 (1996).
12. *The QIA expressionist*, fourth edition, 2000, 51-59, 68, 76, 89.
13. J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Plainview, New York, 1989 (chapter 6).
14. G. T. Hermanson, *Bioconjugate Techniques*, Academic Press, London (UK), 1996 (chapter 3).
15. M. Bruchez-Jr, M. Moronne, P. Gin, S. Weiss and A. P. Alivisatos, *Science* **281**, 2013 (1998).
16. W. C. W. Chan and S. Nie, *Science* **281**, 2016 (1998).